

# Final Report: 0412674

**Final Report for Period:** 08/2005 - 09/2010

**Submitted on:** 11/17/2010

**Principal Investigator:** Snell, Terry W.

**Award ID:** 0412674

**Organization:** GA Tech Res Corp - GIT

**Submitted By:** Snell, Terry - Principal Investigator

**Title:**

BE/GenEn: A Biochemical, Genetic, and Genomic Investigation of the Evolution and Ecology of Sexual Reproduction

## Project Participants

### Senior Personnel

**Name:** Snell, Terry

**Worked for more than 160 Hours:** Yes

**Contribution to Project:** organization, coordination, writing, planning, protein biochemistry, RNAi

**Name:** Mark Welch, David

**Worked for more than 160 Hours:** Yes

**Contribution to Project:** writing, bioinformatics, sequencing, library preparation

**Name:** McArthur, Andrew

**Worked for more than 160 Hours:** No

**Contribution to Project:** Andrew left MBL and has been pursuing other interests

**Name:** Kubanek, Julia

**Worked for more than 160 Hours:** Yes

**Contribution to Project:** analytical biochemistry

**Name:** Hagiwara, Atsushi

**Worked for more than 160 Hours:** No

**Contribution to Project:** Collaborated with the generation of the *Brachionus plicatilis* EST library

**Name:** Serra, Manuel

**Worked for more than 160 Hours:** Yes

**Contribution to Project:** collaborator from the University of Valencia, Spain, modeling, simulation

**Name:** Dingmann, Brian

**Worked for more than 160 Hours:** Yes

**Contribution to Project:** PI on a supplemental award to U Minn Crookston to investigate an in vivo expression system for rotifer genes

**Name:** Morgan, Mike

**Worked for more than 160 Hours:** Yes

**Contribution to Project:** PI on a supplemental award to Berry College to examine differential gene expression between amictic and mictic females

**Postdoc**

**Graduate Student**

**Undergraduate Student**

**Technician, Programmer**

**Other Participants**

Visiting Researchers:

Daniel Robles	Visiting Ph.D. student, Univ of Aguascalientes, Mexico, Mar 09 – Mar 10
Dr. Jiaxin Yang	Visiting Scientist, Nanjing Normal University, April 09 – Mar 10

**Research Experience for Undergraduates**

**Organizational Partners**

**Marine Biological Laboratory**

Co-PI David Mark Welch is a staff scientist at MBL and maintains a lab there. He did most of the gene cloning and sequencing there.

**Nagasaki University**

Scientists at this university collaborated in generation of *Brachionus plicatilis* cDNA library

**University of Valencia**

Scientists at this university collected samples, performed experiments, and analyzed data in support of our project.

**U Minnesota Crookston**

Scientists at this university developed an in vivo gene expression system for rotifer genes

**Berry College**

Scientists at this university analyzed differential gene expression for comparing amictic and mictic females

**Other Collaborators or Contacts**

**Activities and Findings**

**Research and Education Activities:** (See the end of the report)

**Findings:** (See the end of the report)

**Training and Development:**

This project provided the first postdoctoral training for Dr. Kristin Gribble at MBL and Dr. Tonya Shearer at Georgia Tech. Another research assistant at MBL, Ms Jennifer Rocca, was trained in molecular genetics, high throughput sequencing, and bioinformatics and will be entering a doctoral program at the University of Texas at Austin this fall. A high school intern, Mr. Hunt Batter, was hosted in the summer of 2007 who assisted Dr. Gribble. One Ph.D. student at Georgia Tech, Elizabeth Paige Stout, has been trained by this project in the broad area of biochemistry, expanding her knowledge and skills to protein separation, purification and analysis. She now is a postdoc at Scripps institution of Oceanography, and is the first author on a PNAS paper published this year. A fourth year GT Ph.D. student Hilary Smith is being trained on this project. She is co-author on 4 biocomplexity papers submitted this year. In addition, 22 Georgia Tech undergraduates have participated in the research project (see below) and 6 undergrads at Berry College were trained in molecular biology techniques. Two high school teachers participated in the GIFT program (Myra Hensley, North Springs HS and Lawrence Adi, Springhill HS), both in Fulton County.

**Georgia Tech Undergraduates**

Name	Degree	Current Position
Daniel Hicks	BS	MS student at Georgia Tech
Laura Courser	BS	Vet School, University of Georgia
Emily Weigel	BS	Ph.D. student at Michigan State
Josh Cone	BS	Employed
Sonny Patel	BS	Employed
David Krapf	BS	Medical school, Mercer University
Monica Huynh	BS	Employed
Sohee Park	BS	Employed
Joseph Bear	BS	Medical school, Mercer University
Lee Echerd	BS	NASA
Jaclyn Arpin	BS	Employed
Danielle Hubard	BS	Vet school, University of Georgia
Michael Cray	BS	Medical school, Mercer University
Jerry Kim	BS	Medical school, Boston University
Kirk Grubbs	BS	Ph.D. program, University of Wisconsin
Will Carter	BS	MD, resident
Audra Payne	BS	Ph.D. program, University of Tennessee
Kristen Marhaver	BS	Ph.D. program, Scripps Inst Oceanography
Clark Montague	BS	Medical school, Medical College of Georgia
Diana Alba	BS	Medical school, Georgetown University
Ashleigh Burns	BS	Graduates in May 2011
Kathryn White	BS	Graduates in May 2011

**Outreach Activities:**

A high school teacher (Myra Hensley) from North Springs HS, Fulton County participated in the research project in the summer of 2007 through the GIFT program. Again in the summer of 2009, high school teacher Lawrence Adi participated. Suga et al. 2008 results were presented to the MIT Knight Science Journalism Fellows (September 2007) and to the Trustees of the Bay and Paul Foundations (November 2007).

## **Journal Publications**

Snell, T.W., J.M. Kubanek, W.E. Carter, A.B. Payne, J. Kim, M. Hicks, C.P. Stelzer., "A protein signal triggers sexual reproduction in *Brachionus plicatilis* (Rotifera)", *Marine Biology*, p. 763-773, vol. 149, (2006). Published,

C.P. Stelzer and T.W. Snell, "Specificity of the crowding response in the *Brachionus plicatilis* species complex", *Limnology and Oceanography*, p. 125-130, vol. 51, (2006). Published,

Shearer TL and TW Snell, "Transfection of siRNA into *Brachionus plicatilis* (Rotifera).", *Hydrobiologia*, p. , vol. , (2007). Published,

Snell TW, J. Kim, E. Zelaya, and R. Resop., "Mate choice and sexual conflict in *Brachionus plicatilis* (Rotifera)", *Hydrobiologia*., p. , vol. , (2007). Published,

Suga, K., D. Mark Welch, Y. Tanaka, Y. Sakakura, and A. Hagiwara, "Analysis of expressed sequence tags of the rotifer *Brachionus plicatilis*", *PLoS One*, p. , vol. , (2007). Published,

Suatoni, E. S. Vicaro, S. Rice, T.W. Snell, and A. Caccone, "Phylogenetic and biogeographic patterns in the saltwater rotifer *Brachionus plicatilis*.", *Molecular Phylogenetics and Evolution*, p. 4025, vol. 101016, (2006). Published,

Kubanek J, Snell TW, Pirkle C., "Chemical defense of the red tide dinoflagellate *Karenia brevis* against rotifer grazing.", *Limnology & Oceanography*, p. 1026-1035, vol. 52, (2007). Published,

Snell, T.W. and N.J.D. DesRosiers, "Effect of progesterone on sexual reproduction of *Brachionus manjavacas* (Rotifera).", *J. Exp. Mar. Biol. Ecol.*, p 104-109. , vol 363. , (2008). Published,

Suga, K., D. B. Mark Welch, Y. Tanaka, Y. Sakakura, and A. Hagiwara. Two circular chromosomes of unequal copy number make up the mitochondrial genome of the rotifer *Brachionus plicatilis*. *Molecular Biology and Evolution*, p. , vol. , (2008). 10.1093/molbev/msn058, Published,

Carmona, M.J., N. Dimas-Flores, E.M. García-Roger & M. Serra., "Selection for low investment in sex in a cyclically parthenogenetic rotifer.", *Journal of Evolutionary Biology*, p. , vol. , (2009). Published, 10.1111/j.1420-9101.2009.01811.x.

García-Roger E.M., N. Dias, M.J. Carmona & M. Serra., "Crossed induction of sex in sympatric congeneric rotifer populations.", *Limnology and Oceanography*, p. 1845, vol. 54, (2009). Published,

Snell, T.W., T.L. Shearer, H.A. Smith, J. Kubanek, K.E. Gribble, D.B. Mark Welch., "Genetic determinants of mate recognition in *Brachionus manjavacas* (Rotifera).", *BMC Biology*, p. 60, vol. 7, (2009). Published,

Snell, T.W., T.L. Shearer, and H.A. Smith., "Exposure to dsRNA elicits RNA interference in *Brachionus manjavacas* (Rotifera).", *Marine Biotechnology*, p. , vol. , (2010). Published, DOI 10.1007/s10126-010-9295-x.

Stout EP, La Clair JJ, Snell TW, Shearer TL, Kubanek J. Conservation of progesterone hormone function in invertebrate reproduction. *Proceedings of the National Academy of Sciences, USA*, p. 11859-11864, vol. 107 (2010), Published.

Gribble, K.E., T.W. Snell, D.B. Mark Welch. Gene and protein structure of the mate recognition protein gene family in *Brachionus manjavacas* (Rotifera). *Hydrobiologia*, p. , vol. , (2010). In press,

Serra, M., H.A. Smith, J.S. Weitz, and T.W. Snell, "Analyzing threshold effects in the sexual dynamics of cyclically parthenogenetic rotifer populations", *Hydrobiologia*, p. , vol. , (2010). In press,

Snell, T.W., "A review of the molecular mechanisms of Monogonont rotifer reproduction", *Hydrobiologia*, p. , vol. , (2010). In press,

Smith, H.A., D.B. Mark Welch, and T.W. Snell. 2010. Molecular evolution of the membrane associated progesterone receptor within the *Brachionus plicatilis* (Rotifera) cryptic species complex. *Hydrobiologia*, p. , vol. , (2010). In press,

Carmona, M.J., N. Dimas-Flores, J. Montero-Pau & M. Serra. 2010. Effect of experimental methodology on estimation of density at sex initiation in cyclically parthenogenetic rotifers. *Hydrobiologia*, in press.

Yang, J. and T.W. Snell. 2010. Effects of progesterone, testosterone, and estrogen on sexual reproduction of the rotifer *Brachionus calyciflorus*. *International Review Hydrobiology*, In press.

Montero-Pau, J., & Manuel Serra. Density-dependent sex induction and the coexistence of identical competing cyclical parthenogens. *BMC Evolutionary Biology*, submitted.

Gribble, K.E. and D.B. Mark Welch. Evolution and role in speciation of the mate recognition protein gene in the cryptic species complex *Brachionus plicatilis* (Rotifera). In preparation.

### **Books or Other One-time Publications**

Mark Welch, D.B., "Monogononta", (2007). Book chapter, Accepted Bibliography: In: McGraw-Hill Encyclopedia of Science and Technology, 10th Edition

Mark Welch, D.B., "Rotifera", (2007). Book chapter, Accepted Bibliography: In: McGraw-Hill Encyclopedia of Science and Technology, 10th Edition

Kubanek J, and Snell TW, "Quorum sensing in rotifers", In: Chemical Communication Among Microbes, p. 453-461, vol. ASM Pre, (2008). Published,

Serra, M. and T.W. Snell., "Sex loss in rotifers", In: Lost sex: The evolutionary biology of parthenogenesis, p. 281-294 , vol. Springer, (2009). Published,

Snell, T.W., "Contact chemoreception and its role in zooplankton mate recognition", In: Chemical Communications in Crustaceans., p. , vol. Springer, (2010). In press,

### **Contributions**

#### **Contributions within Discipline:**

The research reported here describes the identification of two new proteins that are key to understanding the evolution of reproductive isolation and speciation in rotifers. The first is a mate recognition pheromone on the body surface of female rotifers that regulates male mating behavior. Identification of this gene will enable us to do phylogenetic studies on variation at this locus and how intraspecific variation is transformed into new species. The second gene we have identified controls the life cycle switch from asexual to sexual reproduction. Having this gene enables us to study the evolutionary forces maintaining both asexual and sexual phases in the life cycle. With this gene, we should be able to develop new insights into the evolution of sex, a long-standing problem in evolutionary biology.

#### **Contributions to Other Disciplines:**

Biologists interested in chemical signaling, whether between cells and between organisms, will benefit from our studies. Our work has produced insight into how changes in protein structure lead to functional differences in mating and

reproduction, topics of general interest in sperm-egg interactions, cell-cell interactions, and endocrinology. We are also producing a molecular tool kit that will enable many biologists to use rotifers as a model organism to investigate a wide variety of phenomena like the evolution of sex, genome architecture in asexuals and the evolution of invertebrates.

**Contributions to Human Resource Development:**

This project has trained 18 undergraduates in molecular techniques. Positive experience with science by undergraduates often motivates them to seek a career in science. Often discoveries made while working on an independent research project is the most exciting experience of their undergraduate career. Two Ph.D. students and two post-doctoral scientists are also being trained. Two high school teachers have participated through the GIFT program and will take these experiences back to their classrooms.

**Contributions to Resources for Research and Education:**

This project has allowed PI and Co-PIs to buy supplies used for undergraduate research projects and to pay undergraduate research assistants for their work. We have been able to attract students into the lab who might otherwise never have discovered their passion for research. Support has been provided for Ph.D students and post-doctoral scientists to continue developing their scientific skills.

**Contributions Beyond Science and Engineering:**

## Findings:

### Outline of Scientific Activities

There were several accomplishments during the grant period 2005-2010. We have developed a workable RNAi knockdown system for *Brachionus manjavacas*, we have successfully knocked down expression of several genes involved in asexual and sexual reproduction, and we have identified the mate recognition signal gene (MRP). The rotifer transcriptome database was made public and will serve as the basis for developing targets for the mixis induction protein and its receptors. Steroid hormones have been implicated as important regulators of rotifer reproduction, specifically progesterone and an unidentified androgen. A progesterone receptor has been isolated and characterized and progesterone has been detected in *B. manjavacas*. Variation in the progesterone receptor in natural rotifer populations has been surveyed. Each of these projects is described below.

### Developing Genomic Resources

**Transcriptome Resources.** All transcriptome resources are available to researchers at <http://gmod.mbl.edu>.

*Brachionus manjavacas* cDNA library. We constructed a normalized cDNA library of *B. manjavacas* and bi-directionally sequenced 19,610 clones, resulting in 4,507 contigs representing 4,291 transcripts. Rarefaction and non-parametric richness estimators indicate that total transcript abundance of the library is ~5,000 and that additional sequencing would not be cost-effective. As expected for a normalized library, transcript abundance is remarkably linear, with more than 90% of transcripts represented 1-10 times and less than 0.5% represented more than 100 times.

Deep sequencing of *B. manjavacas* ESTs. We prepared expressed sequence tags (ESTs) from *B. manjavacas* and sequenced their 5' ends using massively parallel pyrosequencing on the Roche GS FLX platform. We obtained 121,894 reads, of which 101,696 passed our in-house quality control standards (Huse et al. 2007). These were assembled into 10,111 contigs or potential transcripts. Despite the short read length, 2,561 contigs had significant matches to the NCBI protein reference sequence database by blastx (E value  $\leq 1 \times 10^{-5}$ ) and 1,988 could be annotated by matches to KEGG or KOG databases. These include transcripts encoding enzymes in the N-glycan biosynthesis pathway, which are likely to be involved in glycosylation of MRP; receptors for mannose and other sugars, which may be involved in male detection of MRP; and proteins that may be part of a progesterone mediated pathway controlling the progression of oocytes into meiosis.

*B. plicatilis* EST and cDNA sequencing. With collaborators at Nagasaki University we sequenced, assembled, and annotated expressed sequence tags from normalized cDNA libraries built from males and from amictic females of *Brachionus plicatilis sensu strictu*. We sequenced 5,254 ESTs from the male library and 3,083 ESTs from the amictic female library representing 4,084 and 2,441 transcripts, respectively.

**Genome Resources.** Fosmid libraries and genome data have been made available to colleagues. Genome sequence data will be available on [gmod.mbl.edu](http://gmod.mbl.edu) when we achieve a stable assembly this fall.

*B. manjavacas* fosmid library. We built a fosmid library of *B. manjavacas* genomic DNA and gridded clones accounting for 3.5x coverage into 384-well dishes and onto multiple sets of nylon membranes. This library was used to isolate the genomic region around the MRP gene, described below. The membranes are available to the community for identifying clones containing genes of interest, and have been used by collaborators at Harvard University and the University of Iowa.

Genome sequencing. We completed low-coverage sequencing projects for *B. manjavacas* and the more distantly related freshwater brachionid *B. calyciflorus* using the Roche GS Titanium platform. The relatively low investment (two runs of the Roche GS per genome) resulted in 25-50% coverage of each genome plus millions of additional bases of unplaced reads that can be used to determine the genomic context of cDNAs, confirm candidate miRNAs (see below) extend PCR products, or identify genes encoding peptide fragments from MS/MS or N terminal sequencing.

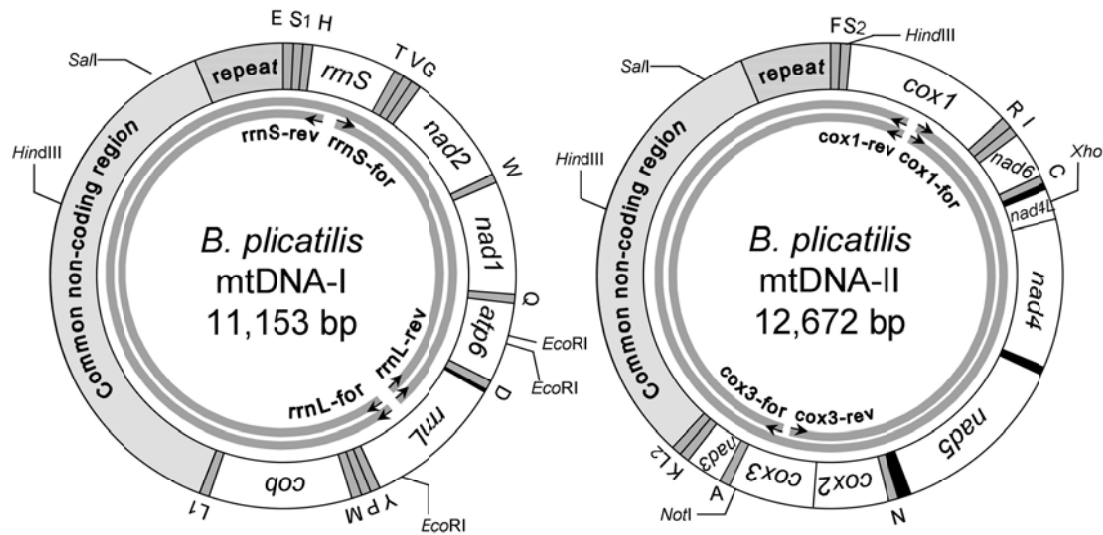
	<i>B. manjavacas</i>	<i>B. calyciflorus</i>
	B. manjavacas	B. calyciflorus
Total reads	1,867,788	2,051,535
Total bases sequenced	534,792,604	765,751,245
Q40+ bases in assembly	42,537,885	82,085,737
Estimated genome size (bp)	225,000,000	153,000,000
Genome at 5x coverage	25%	50%

#### The *Brachionus* mitochondrial genome

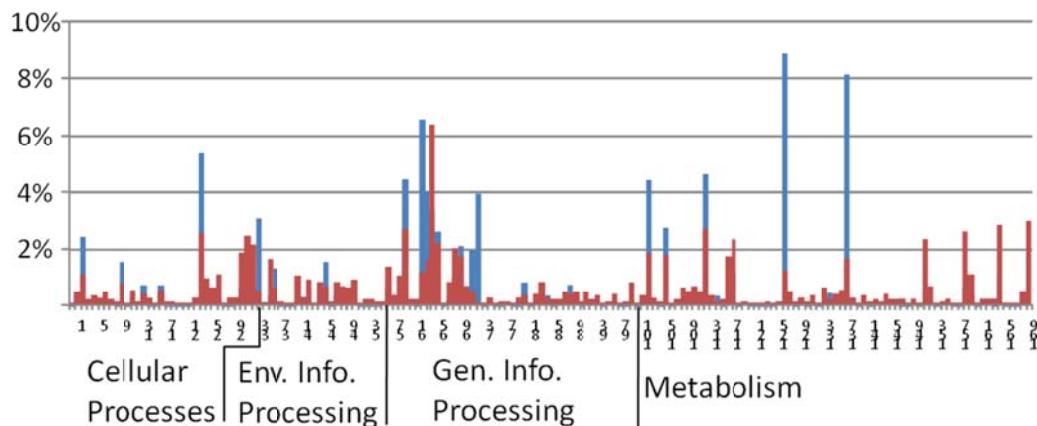
With our collaborator Atsushi Hagiwara at Nagasaki University, we sequenced, assembled, and analyzed the complete mitochondrial genome of *B. plicatilis sensu strictu*. Surprisingly, the genome is organized as two circles of unequal copy number. This was the first description of a stable bipartite mitochondrial genome in a metazoan. Our genome sequencing described above indicates *B. manjavacas* has a very similar mitochondrial genome structure but that of *B. calyciflorus* may be composed of three circles. The recent publication of the mitochondrial genome of the bdelloid Rotaria, as well as unpublished results from our collaborator M. Meselson at Harvard University, indicate that bdelloid rotifers have conventional single-circle genomes. Thus, the evolution of this unique genome structure appears to be specific to



monogononts.



**Analysis of *B. manjavacas* transcripts.** The figure below displays the relative abundance of contigs from the normalized *B. manjavacas* library (red) and the *B. manjavacas* EST library ESTs (blue) for different KEGG functions and shows an excess of ESTs for certain functions.



Analysis of our *B. manjavacas* cDNA library and EST library has revealed several significant features of the transcriptome: 1) There is an identical 33 bp leader sequence on the 5' end of ~6% of transcripts, consistent with a trans-splice leader modification. Such a modification has been observed in a small number of transcripts in bdelloid rotifers (Pouchkina-Stantcheva et al. 2005); however, the 33bp *B. manjavacas* sequence and the 21bp bdelloid sequence have no detectable homology. 2) There is evidence of extensive editing of both 5' and 3' UTRs, seen in contigs with identical coding regions but numerous differences in 5' and/or 3' non-coding regions verified by examination of chromatograms. This editing may be related to regulation of transcript processing by micro RNAs (see below).

The figure below shows four contigs with identical open reading frames encoding the 40S ribosomal protein S21. The start codon is at position 73.

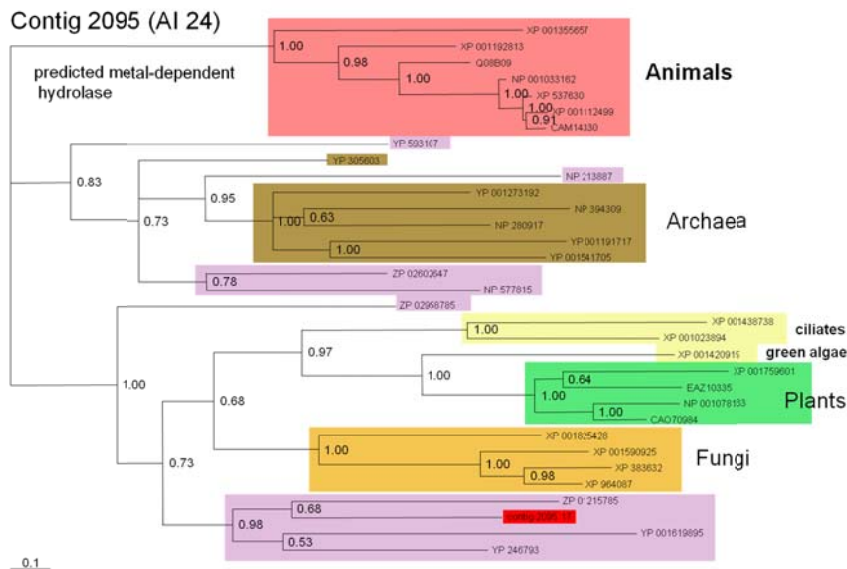
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      10      20      30      40      50      60      70      80      90     100
contig_1110  .GACT--TTAAAGTTTATAACTTAAATTAAAGAAATGATTAAATTTGTCGATCAATAAATTTTCGAATCAAAATGCAAAACGATAAAGGCGAATACACCG
contig_1109  .GAATTAAATAA-ACTTAATAACTTTATTAAAGAAATGATTAAATTTGTCGATCAATAAATTTTCGAATCAAAATGCAAAACGATAAAGGCGAATACACCG
contig_1111  .GACTAATTAAATTTT-TAA--TAATATACGAAATGATTAAATTTGTCGATCAATAAATTTTCGAATCAAAATGCAAAACGATAAAGGCGAATACACCG
contig_4055  .GGGTTTATAA-ATTCAAAACTTTTTTTAAACAAATTTGGTTAAATTTTTCGGTCCATAAATTTTGGATCAAAATGCAAAACGATAAAGGCGAATACACCG

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3) There is evidence of horizontal gene transfer of non-metazoan genes. The presence of genes of bacterial, fungal, or plant origin in the genomes of bdelloid rotifers (Gladyshev et al. 2008) led us to survey the transcriptome of *B. manjavacas* for these “alien genes” using the same methodology (briefly, the difference of the best metazoan blast hit to the best non-metazoan blast hit expressed on a log scale). We found several cases of transcript with an “alien index” of 10-50; all of these transcripts were polyadenylated and thus unlikely to be contaminants. Phylogenetic analysis of the predicted protein products showed that their closest relatives were bacteria or archaeal; some such as a potential D-alanyl-D-alanine dipeptidase had no known metazoan homolog. The presence of an actively transcribed gene with an open reading frame encoding a protein involved in synthesis and catabolism of the bacterial cell wall is particularly intriguing.

While the *B. manjavacas* transcriptome contains many genes of apparently non-metazoan origin both number of genes and their alien index score (in part a measure of how similar they are to extant non-metazoan sequences) are lower than in bdelloids. This suggests that horizontal gene transfer from non-metazoans may have been relatively common in the



common ancestor of bdelloids and monogononts but that the lineage leading to *B. manjavacas* has had a reduced rate of transfer in recent evolutionary history, due to changes in biology and/or habitat.

The figure shows a Bayesian phylogeny of the top blast hits from animals (pink), plants (green), fungi (orange), protists (yellow), archaea (brown) and bacteria (purple) to a transcript from the *B.*

*manjavacas* cDNA library (red). While bacteria are scattered the monophyly of each of the other groups is very well supported. The position of the *B. manjavacas* sequence within a clade of bacteria is strongly supported.

Conserved miRNA    Read  
Count

**micro RNAs of rotifers.** Graduate student Anupriya Dutta has begun investigating the evolution and role of miRNA in rotifers. In collaboration with Kevin Peterson at Dartmouth College, she surveyed the miRNAs in *B. manjavacas* and in the

let-7	4,716
miR-87	4,096
miR-184	804
miR-206	1,377

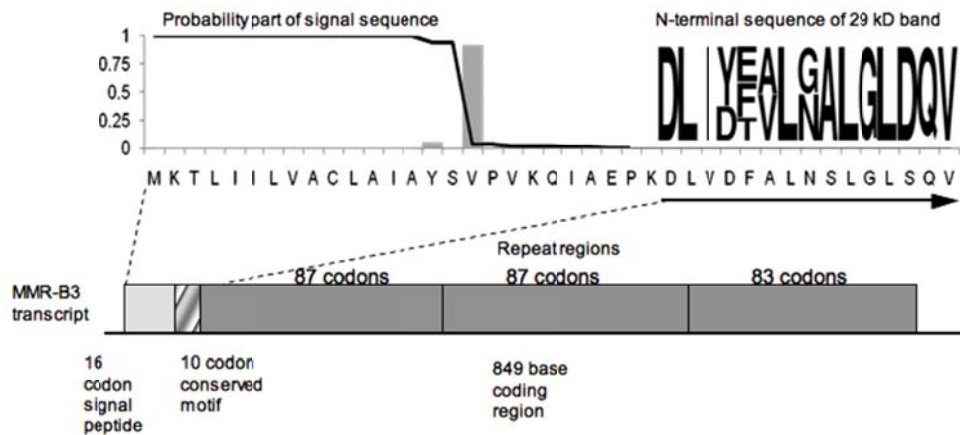
bdelloid rotifer *P. rapida*, and found that monogononts appear to have a greatly reduced repertoire of miRNA types and that the two rotifer groups share very few miRNA classes. The apparent loss of most conserved miRNA types from *B. manjavacas* implies a dramatic change in post-transcriptional gene regulation, involving either novel miRNA types or a different pathway. We have confirmed our initial results with quantitative PCR and preparing miRNA libraries of additional rotifer species for sequencing on an Illumina platform this fall.

## Structure, evolution, and role in speciation of the mate recognition pheromone (MRP) gene

The evolution of chemically mediated pre-zygotic barriers to reproduction plays an important role in speciation. Despite their importance, few genes involved in mate choice have been identified in metazoans, and their evolutionary dynamics remain largely unknown. In the monogonont rotifer *Brachionus*, a cyclical parthenogen, the mate recognition protein (MRP) is a glycoprotein on the body surface of females that is used by males to distinguish compatible mates. Males exhibit a strong preference for young, conspecific females. In this study, we characterized the gene encoding for MRP in *Brachionus manjavacas*, elucidated the evolution of the gene within the *Brachionus plicatilis* cryptic species complex, and employed behavioral assays to quantitatively compare mate choice (isolation distance) with *mrp* sequence and to identify specific nucleotide changes that may be crucial in behavioral reproductive isolation.

### Gene and protein structure and evolution

Using the N-terminal amino acid sequence of the 29 kDa *B. manjavacas* MRP, we obtained a partial sequence of *mrp* from a cDNA library. By sequencing fosmids containing *mrp* from a *B. manjavacas* genomic DNA library, and through analysis of transcripts in *Brachionus* spp. cDNA libraries, we found a family of extracellular matrix protein genes, called MRP Motif Repeat (MMR) genes, with no known homologs. MMR family genes share the same basic structure: a signal peptide sequence, followed by two to seven nearly identical repeat sequences of 276 bp (*mmr-a*) or 261 bp (*mmr-b*), with a truncated final repeat.

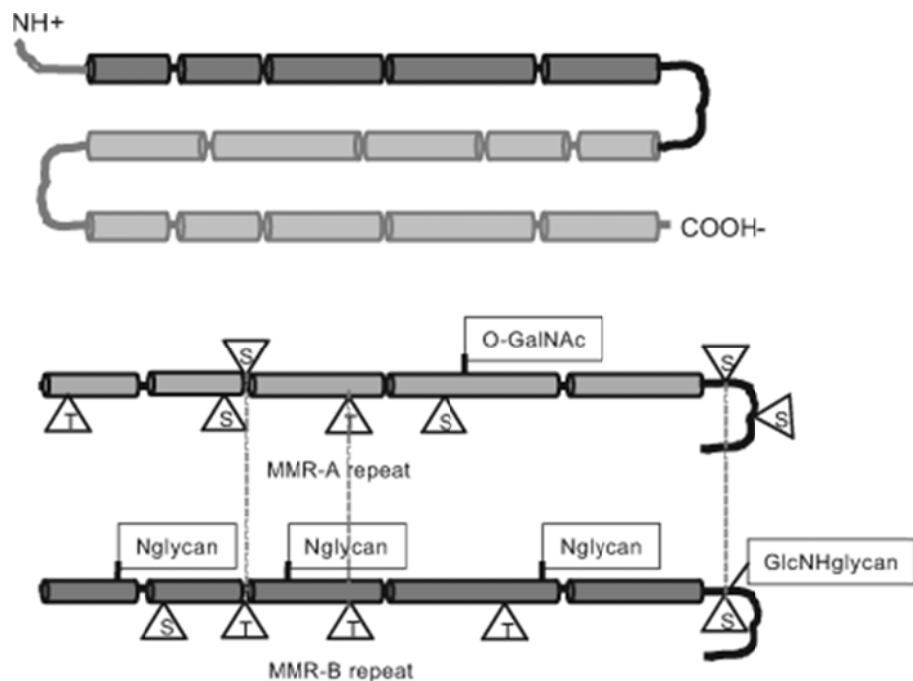


Snell et al. 2009

**Properties of *mmr-b3*.** The gene transcript (bottom) is composed of an 11 base 5'-end untranslated region (UTR), an 849 base coding region (rectangle), and a 34 base 3'-end UTR. The coding region begins with a 16 codon region encoding a signal peptide (light gray box) followed by 10 additional codons (hashed box) before the first of three repeats of a conserved motif (dark gray boxes). The first two motifs are 87 codons each and differ at a single synonymous position; the third motif is truncated after codon 83 by a stop codon (TAA) and differs from the second by a single synonymous position. The 3'-end UTR bears no resemblance to the remainder of the motif. The first 41 amino acids of the predicted peptide (top) show the probability of each residue being part of a signal sequence (line) and the probability of each residue being on the carboxy side of the signal peptide cleavage site (histogram). The sequence logo to the right shows the N-terminal sequence of the 29 kD band, scaled to the frequency of each residue.

Each repeat of the predicted MMR-A and -B proteins is expected to have a secondary structure of 5  $\alpha$ -helices, ranging in length from 11-20 amino acids, separated by coils of 1- 3 amino acids. Hydrophobic and hydrophilic amino acids are predicted to be partitioned to opposite sides of each  $\alpha$ -helix, suggesting that MMR proteins are globular with a hydrophobic core. MMR-A and MMR-B proteins vary in their post-translational modifications, resulting in differences in size and charge, and likely causing differences in the physical properties of the proteins on the surface of the female, and their ability to be recognized by a receptor on a male rotifer.

The potential glycosylation sites are all predicted to face outward, making them accessible to recognition by a putative receptor on the male rotifer. The serine O-linked N-acetylgalactosamine of MMR-A may function as a component of surface mucosal secretions. The function of glycosylation in MMR-B is unclear. The multiple glycosylation sites could be presented on the animal surface in a variety of forms—with multiple mannose residues, or elongated with a variety of monosaccharide chains. We theorize that non-synonymous nucleotide differences between clades in the *B. plicatilis* species complex result in changes in the conformation of MMR proteins, particularly in the relational orientation of glycans, allowing species differentiation by males.



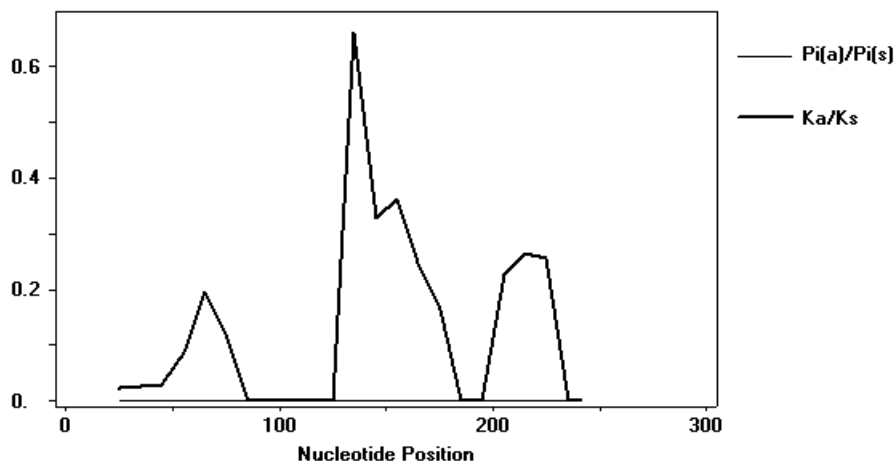
**Structure of MMR Proteins.** A) Cartoon of the proposed secondary structure of MMR-B3; the first MMR is darkened. Other MMR proteins would have the same general structure, differing in the number of MMRs. B) Potential post translational modifications on MMR-A and MMR-B repeats. O-GalNAc: O-linked N-acetylgalactosamine attachment site; GlcNHglycan: glycosaminoglycan attachment site; N-Glycan: N-glycosylation site; T, S: sites of threonine or serine phosphorylation. Vertical lines connect similar modification sites on the two gene types.

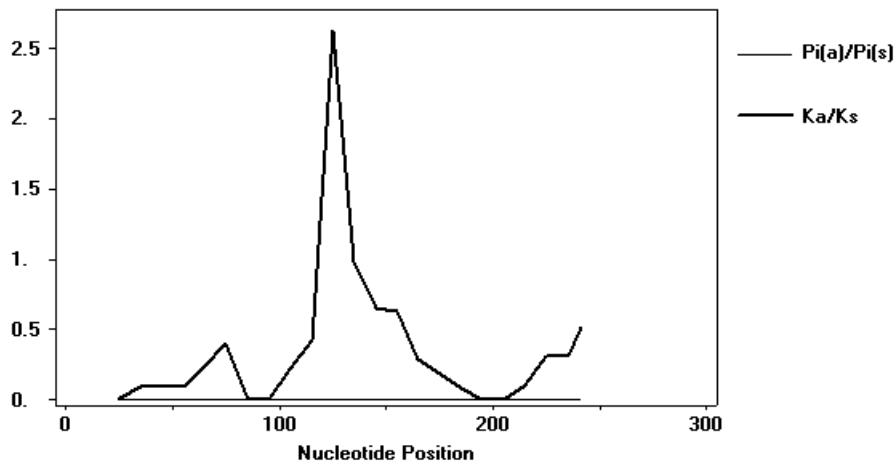
Synonymous divergence ( $K_s$ ) between A and B repeats suggests that the two gene classes arose from a duplication event many millions of years ago. Few nuclear protein coding genes have yet been sequenced in multiple monogonont species; however,  $K_s$  of the widely sequenced mitochondrial gene *cytochrome oxidase I* is effectively saturated between the *B. plicatilis* species complex and other brachionids (*B. calyciflorus*, *B. havanaensis*, *B. quadridentatus*, *B. rubens*, *B. urceolaris*), suggesting that the duplication may be specific to the *B. plicatilis* species complex.

The *mmr-a* and the *mmr-b* repeats are likely kept homogeneous through concerted evolution, a process of non-independent evolution of repetitive DNA sequences mediated by unequal crossing over and/or gene conversion, in a manner similar to that employed in the conservation of identity in ribosomal protein genes (Vacquier, 1995; Horth, 2007). This can have a stabilizing effect on the protein sequence, as new mutations are likely to be lost. More rarely, however, the same process of concerted evolution can rapidly spread the mutation across all of the repeats. When this results in conformational changes in the protein detectable by males, it could lead to reproductive isolation between formerly compatible species, and thereby to speciation.

The repeat motifs and the arrangement of two copies of *mmr-a* only 7590 bp apart on a single chromosome in *B. manjavacas* fit a pattern seen in mating genes across eukaryotes. In organisms including algae, yeast, plants, and abalone, genes involved in mate recognition are frequently organized as a series of tandem repeats, with duplicated genes found in clusters (Vacquier, 1995; Ferris et al., 1997; Mayfield et al., 2001; Bennett et al., 2003). These sex-related genes undergo more rapid evolution than other protein-coding genes, though the reason for, and mechanism behind, this is not clear.

Regions of *mmr-b* appear to be under positive and non-conservative selection. In a sliding window analysis comparing the repeats between pairs of isolates,  $K_a/K_s$  shows three peaks, a trend that holds in comparisons across the species complex.  $K_a/K_s$  is a maximum of 0.65 between two *B. plicatilis* sensu strictu isolates, and reaches as high as 2.5 in the same region of the gene between isolates from the more distantly related A and B clades of the species complex.



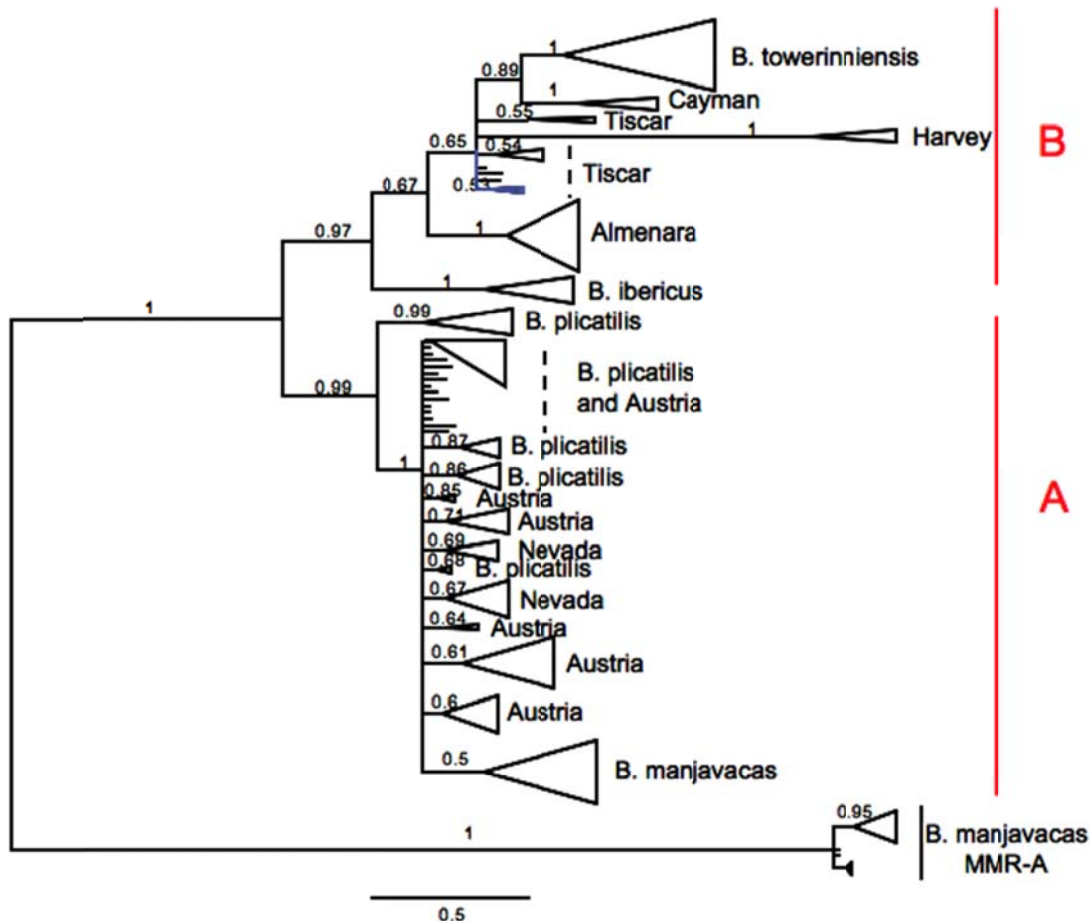


**Ka/Ks shows regions of positive selection along *mmr-b* repeat between isolates from *Brachionus plicatilis* complex.** Top: Ka/Ks between two isolates of *B. plicatilis* sensu strictu. Top: Ka/Ks between isolates from the more distantly related A and B species clades.

### Phylogeny of MMR repeats in *Brachionus*

We identified one of the MMR genes, *mmr-b*, as the gene for MRP through knockdown by RNAi, as described above. We sequenced *mmr-b*, from 24 isolates representing 11 phylotypes within the *B. plicatilis* species complex and found from one to four copies for each isolate, ranging in length from two to nine repeats. The phylogeny of *mmr-b* recapitulates that of the species complex as determined by the phylogenies of *cox1*, *its1*, and *hsp82*, but with longer branch lengths indicating more rapid evolution of *mmr-b*, as expected for genes involved in sexual reproduction.

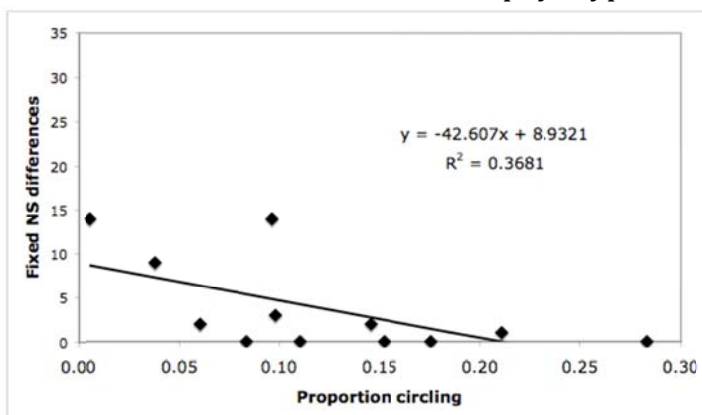
Sequences of *mmr-b* fell into two main clades, one containing isolates of “Clade B” phylotypes, *B. ibericus*, Almenara, Tiscar, Harvey, Cayman and Towerinniensis, and the second containing “Clade A” isolates, *B. plicatilis* sensu strictu, Nevada, Austria, and *B. manjavacas*. Sequences within the “B” clade were monophyletic for each phylotype, except for those of Tiscar. The very long branch for the Harvey phylotype suggests extremely rapid evolution of *mmr-b* within this isolate. Within the “A” clade, sequences from the various phylotypes were largely unresolved. Of particular interest were sequences for *B. plicatilis* sensu strictu, which fell into two separate clades, each represented by three isolates. The divergence of the gene within a single species could indicate emerging reproductive isolation.



**Phylogeny of *mmr-b* within the *B. plicatilis* species complex.** Numbers indicate Bayesian posterior probabilities.

## MRP and reproductive isolation

To examine the relationship between *mmr-b* divergence and behavioral reproductive isolation, we carried out reciprocal crosses between pairs of isolates from different *mmr-b* phylotypes, and quantified the percentage of male-female encounters that resulted in mating. The estimated degree of reproductive isolation was positively, though not statistically significantly, correlated with *mmr-b* sequence distance. Thus, there appears to be a gradient in mate recognition within the cryptic species complex. Isolates of different phylotypes within clade A showed varying but positive levels of mate recognition, while crosses between isolates from clades A and B or crosses between phylotypes within clade B did not result in mating.



**Correlation between *mmr-b* divergence and mate recognition.**



The mating bioassays point to the powerful role MRP likely plays in the formation of new species. In a cross of two isolates of *B. plicatilis* sensu strictu with divergent *mmr-b* sequences, the males of one isolate fail to recognize the females of their own strain, and instead show a preference for outcrossing with females of the other, more ancestral *mmr-b* type. Similarly, the fast-evolving, long-branch Harvey strain shows an extremely low level of self-recognition and does not appear to outcross with other species from Clade B. These results provide evidence of incipient speciation—as MRP rapidly evolves, we would expect that males would no longer recognize conspecific females. The new version of MRP could be propagated through a population via asexual reproduction, and over time drift would lead the reproductively isolated population to become a new species.

The *B. plicatilis* complex carries several of the signatures expected for speciation caused by sexual selection: greater species richness; closely related species that differ in mating signals and preferences but that differ little in other traits; partial pre-zygotic isolation between populations; and asymmetry in mate preferences between males and females of different populations. We hope to continue to use this developing model system to address questions about post-zygotic reproductive isolation and the specificity of the induction of sexual reproduction.

### **Techniques for RNAi in Rotifers**

RNA interference (RNAi) is a powerful technique for functional genomics. Yet no studies have reported its successful application to zooplankton. Many zooplankton, particularly the microscopic metazoans of phylum Rotifera, have unique life history traits for which genetic investigation has been limited. RNAi is accomplished in rotifers by the exogenous introduction of double-stranded RNA (dsRNA) through use of a lipofection reagent.

Expression of three genes in the pathway for progesterone-mediated oocyte maturation was significantly suppressed in the rotifer *Brachionus manjavacas*, including heat shock protein 90 (hsp90), progesterone receptor, and mitogen activated protein kinase (MAPK). Transfection with dsRNA for each gene significantly increased the proportion of non-reproductive females, more than doubling the percentage in the hsp90 and MAPK treatments. Additionally, a fluorescence-based lectin binding assay confirmed significant suppression of four of six glycosylation enzymes that were targeted with dsRNA. Successful suppression of mRNA transcripts was confirmed with qPCR.

Development of RNAi for rotifers promises to enhance ability for assessing genetic regulation of features critical to their life history, and represents a key step toward functional genomics research in zooplankton. Moreover, use of lipofectants for transfection is a technique hitherto primarily applied to cultured cells. Demonstration of their use in an invertebrate suggests potential for lipofection-mediated dsRNA delivery to permit RNAi in taxa previously considered unreceptive to environmental RNAi.

### **Molecular evolution of the membrane associated progesterone receptor**

Many studies have investigated physiological roles of the membrane associated progesterone receptor (MAPR) and its homologs in taxa ranging from yeast to mammals, but little is known regarding the evolution of the gene. Marked differences in the effects of exposure to exogenous progesterone on resting egg production of four brachionid rotifers have been reported, suggesting differences in progesterone signaling and reception among these congeneric species. The sequence similarity for the *MAPR* among members of the *Brachionus plicatilis* species complex is described. Phylogenetic analysis of this receptor was compared with prior studies of relatedness based on ITS and CO1 sequences. Analysis of ratios of non-synonymous to synonymous site substitutions ( $K_a/K_s$ ) was used to assess molecular evolution of the gene among several brachionid clades. This study advances understanding of molecular evolution of the rotifer *MAPR* gene and further defines the phylogenetic relationship among cryptic species of the *B. plicatilis* species complex.

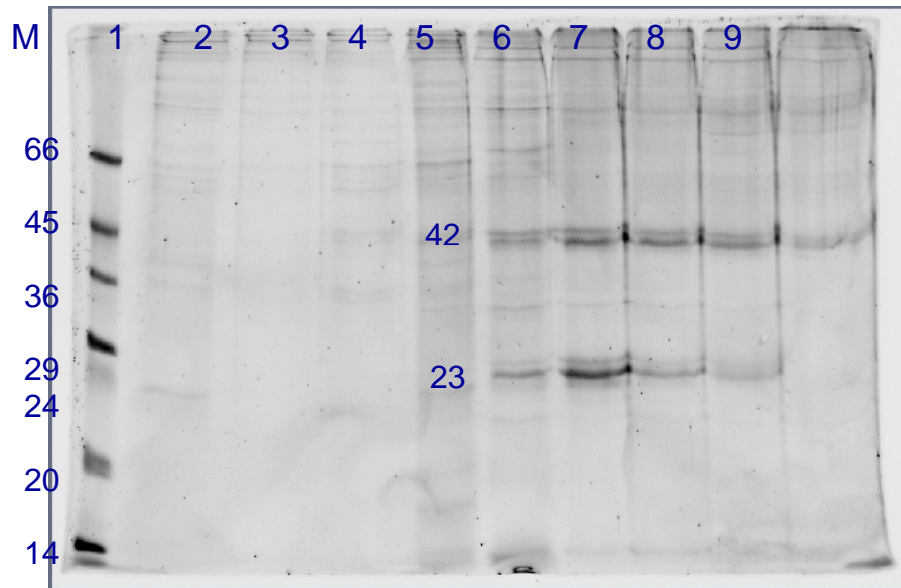
### **Chemical signals regulating monogonont rotifer reproduction**

A review was written of the chemical signals regulating rotifer reproduction. Most animals use proteins, peptides, steroids, eicosanoids, or amino acid derivatives as chemical signals, along with receptors, secondary messengers, transduction systems, and transcription factors to finely control reproduction. Many protostomes have complex endocrine systems with vertebrate-like sex steroid receptors, but some are unresponsive to vertebrate sex steroids. Others are responsive to estrogen and testosterone, but the effects are mediated through non-estrogen receptor pathways. The pheromones that rotifers use to synchronize reproduction were reviewed, as well as rotifer responses to waterborne vertebrate steroid hormones. The impact on rotifer reproduction of endocrine disruptors that mimic androgens and their antagonists were described. A fraction of the brachionid transcriptome was surveyed for candidates involved in endocrine signalling systems and genes were identified that are putatively involved in steroidogenesis and oocyte maturation. RNAi was used to selectively knock down gene expression and identify the functional roles of genes hypothesized to be involved in the regulation of rotifer reproduction.

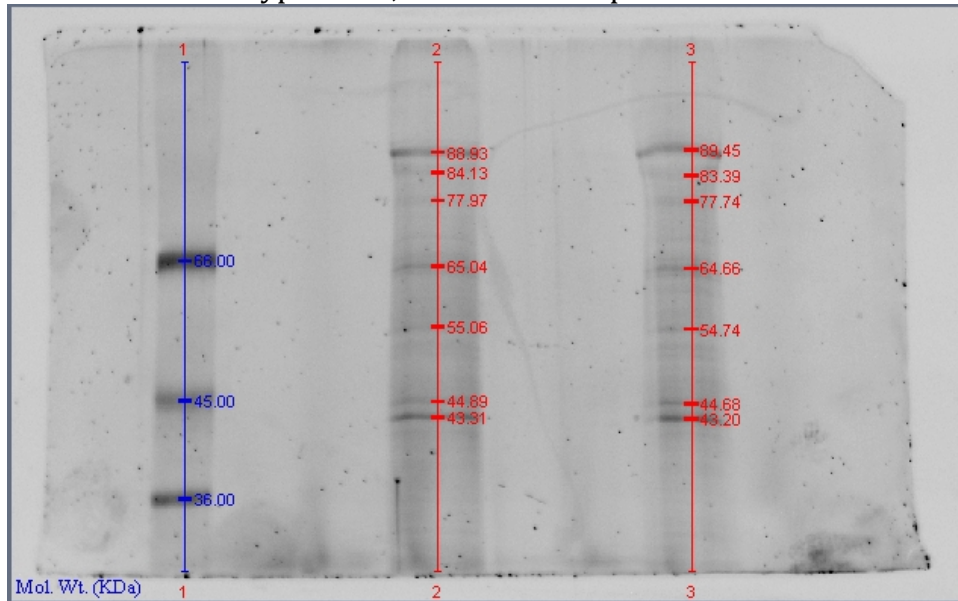
### **Sequencing of Proteins in Rotifer Conditioned Medium**

We purified the proteins in rotifer conditioned medium using a variety of chromatographic methods. Fractions were bioassayed for mixis-inducing activity and subjected to polyacrylamide gel electrophoresis (Figures 1&2). Some samples were sent to the University of Michigan Proteomics Center for *de novo* sequencing by mass spectrometry and others were sent to Emory University's microchemical facility for N-terminal sequencing. In addition to the mixis inducing protein (MIP), we are looking for other rotifer proteins that may have an interesting role in regulating the rotifer life cycle.

The MIP is secreted by rotifers into surrounding water, thus conditioning the aqueous medium of a dense rotifer culture. By monitoring rotifer population densities, it was determined that 1 rotifer per 15 ml water is the threshold concentration at which mixis is induced (Snell et al 2006). We used amino acid sequences from rotifer proteins found in conditioned medium (Table 2) to design degenerate primers to amplify genes involved in mixis. We tested rotifers transfected with dsRNA for the genes of interest and evaluated mixis rates in a bioassay. We have screened several genes (~12) and found two whose knockdown significantly suppresses mixis.



**Figure 1.** Proteins accumulating in rotifer conditioned medium over 9 days. M represents the molecular weight markers, and lanes 1-9 represent medium harvested on those days of the rotifer culture. Based on our hypothesis, 23 and 42 kDa proteins became candidate MIPs.



**Figure 2.** Proteins from rotifer neonate conditioned medium (before feeding). Lane 1 represents the molecular weight markers, and lanes 2 and 3 are identical, representing neonate conditioned medium. Peptide sequence was obtained for 3 proteins, 65, 44, and 43 kDa.

Protein	Partial Sequence
89 kDa	DASYVDER
65 kDa	RDI/L[*]SI/LPR, where * is GN, NG, OR GGG
	I/LDVI/LEGNEQF/met-ox
45 kDa	[*]AAGNNI/LDENR, where * could be RN, NR, RGG, GGR, OR GRG
43 kDa	I/LN[*]Q/KFETDGSAR, where * could be GI/L, I/LG, AV, OR VA

	[*]EI/LNGI/LTVAAR, where * could be AP or PA
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**Table 1.** Partial amino acid sequences from de novo mass spectrometry sequencing of proteins in rotifer conditioned medium from neonates.

Protein	Partial Sequence
16 kDa , plastocyanin p = 0.29	aVIKLGSDsGALVFEPXXv
25 kD, chlorophyll a/b/ binding protein p = 0.19	vEFYGPDRAXLGPf
39 kD, steroidogenesis inducing protein	dVNGGGATLPQpLYQTA
42 kD, putative transcriptional regulator, p = 1.00	dITLTGEAQmgViye

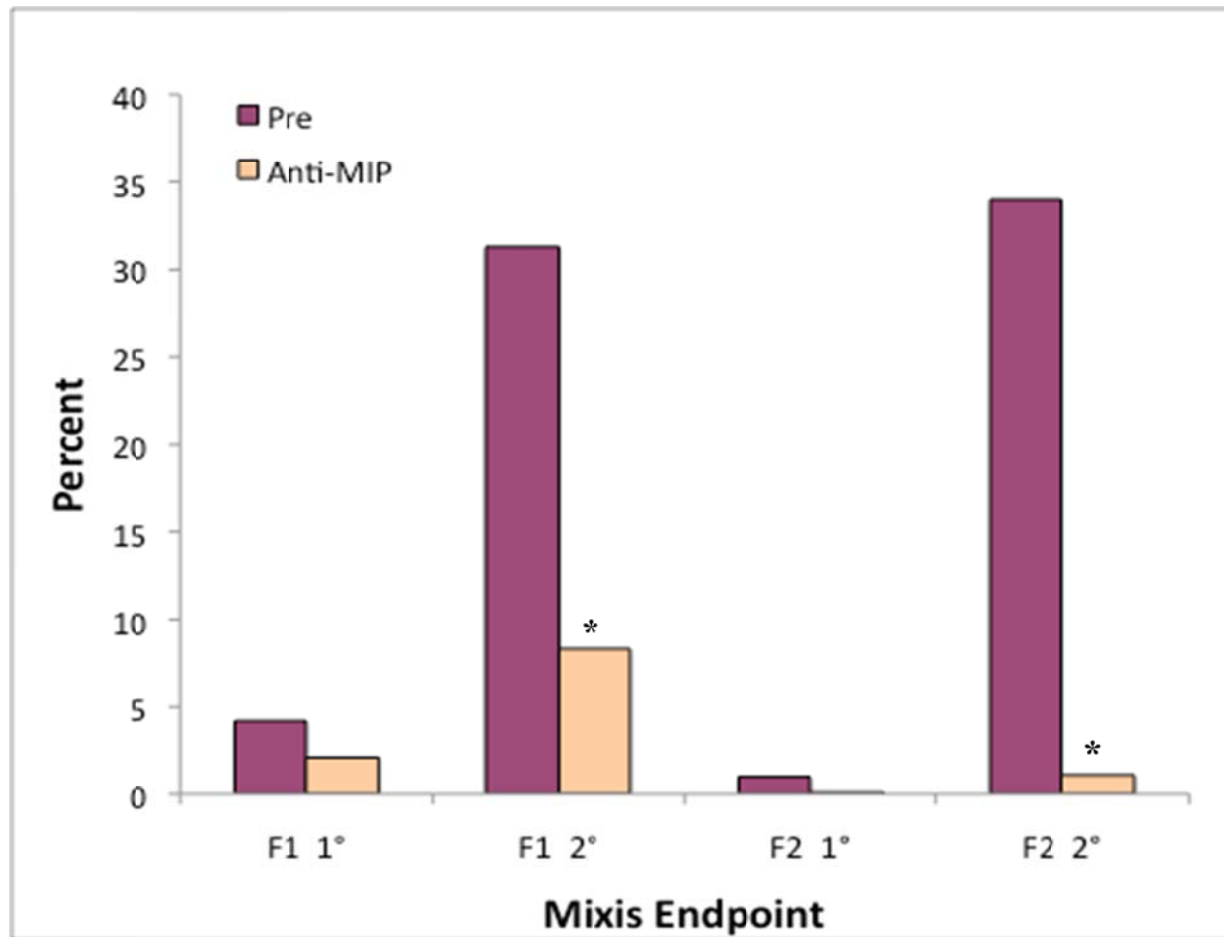
**Table 2.** Partial amino acid sequences from N-terminal sequencing of proteins in rotifer conditioned medium from mass cultures.

### Anti-MIP antibody

A rabbit polyclonal antibody was prepared with a synthetic peptide from the N-terminal sequence of the 39 kD protein isolated from rotifer conditioned medium. The 17 amino acids from the putative MIP (dVNGGGATLPQpLYQTA) were conjugated to keyhole limpet hemocyanin to render the protein more immunogenic. In a Western blot, this antibody binds to four major proteins in rotifer conditioned medium, 230, 189, 134, and 67 kD. Also in a Western blot, this antibody binds to two major proteins in rotifer homogenate, 178 and 130 kD. When the MIP antibody is used to immunoprecipitate rotifer conditioned medium, only one major protein of 22 kD is detected. This band was cut from a PAGE gel and subjected to tryptic digest followed by mass spectrometry. Peptides from five proteins were detected in the 22 kD band.

Protein	Rotifer species
Plasma membrane calcium-dependent ATPase	Philodina roseola
Tankyrase-like protein	Adineta vaga
Na/K ATPase alpha subunit	Brachionus plicatilis
Triosephosphate isomerase	Philodina roseola
Exportin-5-like protein	Philodina roseola

When the MIP antibody is placed in the medium of a mixis bioassay, females are less likely to become mictic (see Figure 3 below). We hypothesize that this is because the antibody binds to the MIP in the medium and prevents this protein from binding to its receptor on females. Females detect a reduced mixis stimulus and thus a lower proportion become mictic.



**Figure 3.** Inhibition of mixis by the anti-MIP. F1 are the progeny of *B. manjavacas* resting egg hatchlings. 1° refers to mixis in the maternal female and 2° refers to mixis in her progeny. Percent on the Y-axis is the percent of 48 females in a mixis bioassay that were mictic. The Pre treatment is plasma from the rabbit prior to immunization, and the Anti-MIP treatment is plasma from the same rabbit after inoculation with the MIP antigen. Asterisks indicate significant differences in Pre and Anti-MIP mixis by Fisher's Exact Test.

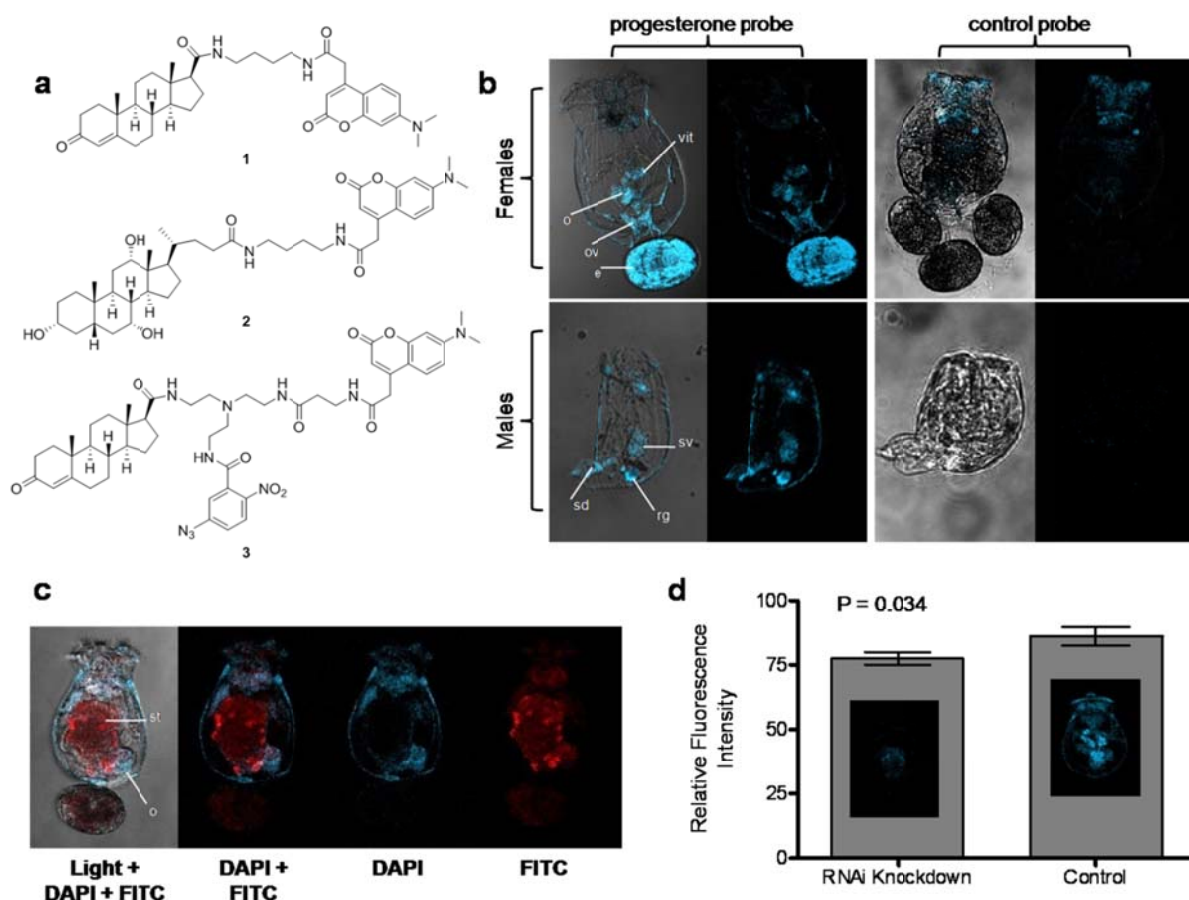
### Identifying Steroids in Rotifers

Our previous work showed that the N-terminus of the rotifer MIP is 100% identical ( $p = 0.00084$ ) to the N-terminal fragment of human SIP protein (P83897) isolated from ovarian follicular fluid (Snell et al. 2006). Since the SIP protein is known to be a steroidogenesis-inducing protein in humans, this suggested that rotifers may be using a similar steroidogenesis-inducing protein to regulate mixis. If true, the direct conclusion is that steroid hormones that regulate reproduction in invertebrates may be more phylogenetically conserved than previously believed. This raises questions about the nature of these steroids and their relationship to the well characterized steroids of vertebrates. We have attacked this problem in two ways: first by extracting steroids from rotifer biomass and chemically characterizing them (see below), and second by searching the rotifer EST library for receptors of known steroids. We have found sequences with strong similarity to both progesterone and androgen receptors of humans in the rotifer transcriptome, whereas receptors for estrogen and testosterone are apparently absent.

A previous study performed by Joaquim-Justo and Snell (2009) showed that sexual reproduction in rotifers was strongly affected by the addition of androgen receptor antagonists, suggesting that sexual reproduction is most likely under endocrine control and that there may be natural androgen-like molecules that regulate sexual reproduction in rotifers. Little is known about how reproduction is regulated in rotifers, or the molecules involved in reproductive signaling in most non-arthropod invertebrates.

We designed and developed a molecular probe to progesterone suitable for both histological analyses in live rotifers and subsequent protein identification studies by immunoaffinity methods. By installing a 7-dimethylamino-4-coumarin tag onto progesterone, the same dye could be used for both *in vivo* imaging and *in vitro* isolation of progesterone receptors in *B. manjavacas*, given ready access to a selective monoclonal antibody (mAb) against the fluorophore (Fig 4a). Incubation of live and dead rotifers with the fluorescent progesterone probe (FPP) consistently resulted in localization of the probe in reproductive organs in both females and males, including the ovaries, vitellarium (yolk glands), oviduct, and egg in females, and the seminal vesicle, rudimentary gut, and sperm duct in males (cf. blue fluorescence in Fig. 4b). Interestingly, eggs attached to female rotifers fluoresced with FPP (**1**) only when punctured, suggesting that the egg shell prevented probe uptake. Using the natural auto-fluorescence of the green alga food *Tetraselmis suecica* (excitation 488 nm), we confirmed that FPP (**1**) was not localizing in the stomach as a result of ingestion (Fig. 4c). Furthermore, male rotifers do not feed and lack stomachs, providing evidence that the staining of male reproductive organs is due to the membrane permeability of FPP (**1**).

Genome-mining of a rotifer cDNA library (rotifer transcriptome sequencing project; <http://gmod.mbl.edu>) led to the identification of a 799 nucleotide sequence (contig\_1830) with strong similarities to various progesterone receptors, including that of the sea urchin *Strongylocentrotus purpuratus*. The presence of a progesterone receptor in *B. manjavacas*, led us to explore the role of endocrine signals in the reproductive physiology of this ancient lineage. We also were encouraged by knowledge that the sex steroid receptor gene family also is ancient, arising early in metazoan evolution and pre-dating the divergence of protostomes and deuterostomes (Kohler et al. 2007). Together, these studies suggest that progesterone is likely a key regulatory steroid involved in the regulation of reproduction in *B. manjavacas*. While the isolation of progesterone was limited by conventional isolation methods, we were able to detect this hormone using progesterone EIA. Using a dual fluorescent and affinity probe, we were also able to demonstrate receptor-selective localization in live *B. manjavacas*. This was confirmed further by RNAi knockdown and affinity experiments, validating the binding of progesterone to its receptor. Our data demonstrate that the well described mammalian steroid reproductive hormone progesterone is also likely to play a central regulatory role in the reproduction of the marine invertebrate, *B. manjavacas*, and that conservation of function occurs over a broad expanse of animal phylogeny.



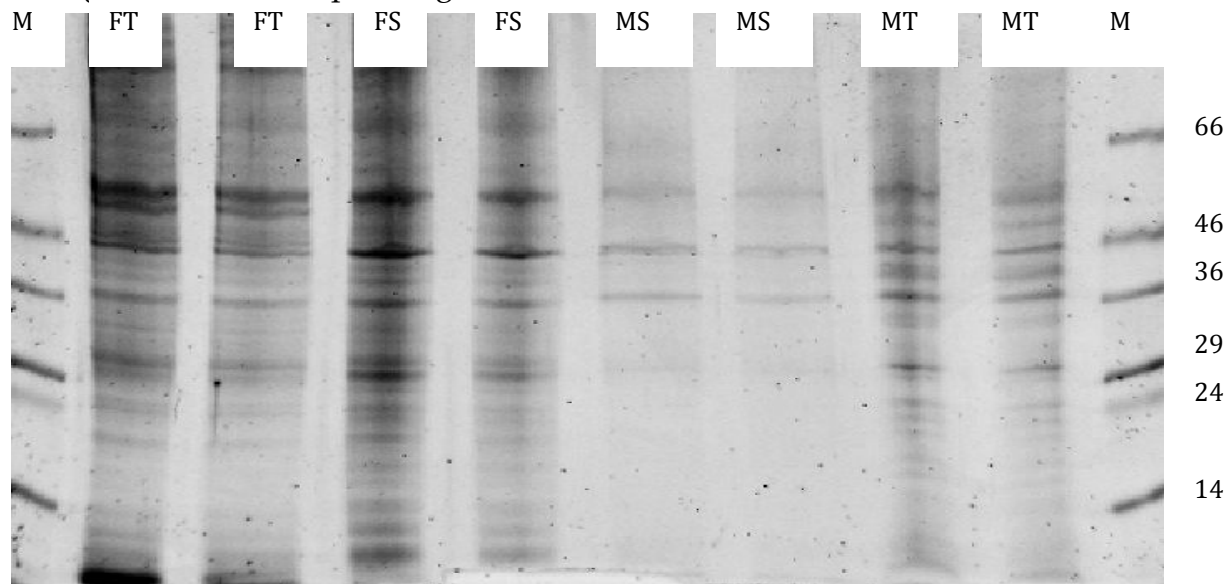
**Figure 4.** (a) Novel fluorescent probes designed to localize, identify, and characterize the progesterone receptor in *Brachionus manjavacus*, fluorescent progesterone probe (FPP, 1), fluorescent control steroid probe (FCP, 2), and photocross-linking fluorescent progesterone probe (3). (b) multiphoton confocal microscopy (MPCM) images of female (10×) and male (20×) rotifers incubated with either FPP (1) or FCP (2) under DAPI fluorescence: vit – vitellarium (yolk gland); o – ovaries; ov – oviduct; e – egg; sv – seminal vesicle; rg – rudimentary gut; sd – sperm duct. (c) MPCM images of female rotifer fed *Tetraselmis suecica* and incubated with FPP (1), *T. suecica* autofluoresces red with FITC, and FPP fluoresces blue with DAPI: st – stomach, o – ovaries. (d) relative fluorescence intensity of female rotifers incubated with FPP (1) after being transfected with with dsRNA representing a bp fragment of the rotifer progesterone receptor gene and female controls (n = 8, two sample t-Test with equal variances, one-tailed P = 0.034).

### Identifying MIP and MRP Receptors on the Body Surface of Males and Females

Receptors for the MIP and MRP proteins are likely to be located on the body surface of female and male rotifers, respectively. We isolated surface proteins from females and males by biotinylating surface proteins with a cell-impermeable biotin. Rotifers were then homogenized and biotinylated proteins isolated on an avidin affinity column. Bound proteins were cleaved from the biotin and collected in the eluate. Male and female surface proteins are compared to the total protein complement of rotifers in Figure 5. Females have many surface proteins (FS lane), but males have only 3 major bands (MS lane) of molecular masses of 36, 45, and 53 kD, one of which is likely the MRP receptor. A Far



Western blotting protocol (Pierce) was used to identify candidates for the MIP receptors. Prey proteins (the surface proteins described above) were separated using PAGE electrophoresis and blotted on a membrane. The membrane was hybridized with biotinylated bait proteins. For example, bait proteins in rotifer conditioned medium (containing the MIP) were hybridized to female surface proteins that contain the putative MIP receptor. Since rotifer conditioned medium contains the MIP, we expected it to bind to its receptor among the female surface proteins. This seemed like a powerful method for identifying good candidates for the MIP receptor. Protein-protein interactions on the membrane were visualized by chemiluminescence using avidin-horseradish peroxidase. We identified six female surface proteins of molecular weights of 87, 75, 54, 48, 42, and 30 kD that bound to proteins in rotifer conditioned medium. We are examining these through amino acid sequencing and RNAi knockdown followed by bioassays.



**Figure 5.** SDS-PAGE gel of female and male surface proteins. M – MW marker, FT – female total proteins, FS – female surface proteins, MS male surface proteins, MT – male total proteins. Number on right are MW in kD.

### Sexual Reproduction in Natural Rotifer Populations in Eastern Spain

One of the objectives of the Rotifer Biocomplexity project is to analyse natural populations for variation in sexual reproduction through the MIP and its receptors. Heritable changes in the investment in sex are expected to occur during the annual population growth, because clones with a high propensity to sex would be selected against. The following five papers explore this problem.

**Sex loss in monogonont rotifers.** We reviewed the literature on the monogonont rotifer life cycle in order to explain how cyclical parthenogenesis is evolutionarily maintained. We also compared monogonont rotifer life cycle with that of their close relatives the bdelloid rotifers, which are ancient obligate asexuals. Our analysis clarifies that the cost of sex in monogononts is two-fold when compared to an obligate asexual lineage on an annual time-scale. However, when compared to an obligate sexual, cyclical parthenogens avoid the cost of sex in every parthenogenetic generation. In monogonont rotifers, where sexual reproduction is triggered



by crowding, reproducible selection for loss of sex has been reported in laboratory experiments. The mechanistic hypothesis is that some obligate asexual clones arise by spontaneous mutation, and they fail to respond to the sex triggering chemical signals produced by conspecifics. Hence, in these clones, asexual females never produce sexual daughters. Using a simple model, we showed that, as a result of the association of sex with dormancy, sex loss results in a huge short-term advantage, because sexual females only produce males or diapausing eggs, and do not contribute to current population growth. However, the requirement of sex for dormancy should result in a mid-term selection pressure to retain sex. It is this mid-term pressure which stabilizes cyclical parthenogenesis and allows its maintenance. From this analysis, the periodic occurrence of obligate asexuals is predicted in monogonont rotifer populations, especially those inhabiting habitats with infrequent adverse periods.

**Selection for low investment in sex in a cyclically parthenogenetic rotifer.** Qualitative predictions the sex loss model described above were tested for field populations. Specifically, we tested whether selection for low investment in sex in rotifers occurs within a single planktonic growing season. We studied the dynamics of the heritable variation in propensity to sexual reproduction among clones of a *Brachionus plicatilis* rotifer population in a temporary Mediterranean pond during its planktonic growing period. Clonal isolates displayed high heritable variation in their propensity to sex. Moreover, the frequency of clones with low propensity to sex increased during the growing season, which supports the hypothesized short-term selection for low investment in sex within a growing season. These results demonstrate (1) the inherent instability of the cyclical parthenogenetic life cycle, (2) the cost of sexual reproduction, and (3) the role of the association between sexual reproduction and dormancy in maintaining sex in these cyclical parthenogens.

**Theoretical analysis of the threshold effects in the sexual dynamics of cyclically parthenogenetic rotifer populations.** Sex in cyclically parthenogenetic rotifers is triggered when a threshold population density is achieved, an effect mediated by the accumulation of a mixis inducing protein (MIP) in the medium. We built models explicitly accounting for MIP dynamics. Model analysis suggests that either (1) a positive feedback of MIP concentration on MIP production or (2) a relationship between the proportion of sexual females and MIP concentration is needed for a threshold in production. Alternatively, a threshold in response could create the nonlinear dynamics observed in mixis induction. To obtain insights into both possibilities, an experiment was performed to estimate the sexual response to a dilution series of medium containing MIP. After regression analysis, results do not allow clear discrimination between both possibilities. However, the observed smooth increase of sexual offspring with MIP concentrations supports the hypothesis of a positive feedback on MIP production, and deserves further investigation.

**Theoretical analysis on how density-dependent sex induction can mediate the coexistence of identical competing cyclical parthenogens.** Only sex-based mechanisms have been shown to create the negative feedback needed for stable coexistence of competitors with identical niches. Increasing evidence of coexistence of cryptic species with no recognized niche differentiation has called attention to these mechanisms. We addressed the hypothesis that density-dependent sexual investment can mediate coexistence of identical-niche cyclical parthenogenetic species. We modeled the dynamics of two competing cyclical parthenogens with species-specific density-dependent sexual investment (i.e., species-specific MIP for

rotifers) and either equal or different competitive abilities. Using symbolic analysis and computer simulation, we found that investment in sexual reproduction creates an opportunity for another species to invade and become established. This may happen even if the invading species is an inferior competitor. Our results can be extended to a broader range of species where similar density-dependent mechanisms could act promoting coexistence.

**Cross induction of sex in sympatric congeneric rotifer populations.** The response to MIP has been hypothesized to be species-specific. If not, the timing of sex and final diapausing egg production of a species might not be optimized. A set of experiments – based on individual and mass-culture approaches – were carried out to investigate the differentiation in sex induction signals between sympatric and allopatric congeneric *Brachionus* populations by analysing the capability of the medium conditioned by a population to induce sex in a different one. We found low specificity in the response to sex inducing signals between some of the populations studied. Differences among species in their sexual response seem to be related to differences in MIP production and sensitivity to MIP. Nevertheless, our results suggest some differentiation between *B. manjavacas* and *B. plicatilis* in their MIP.

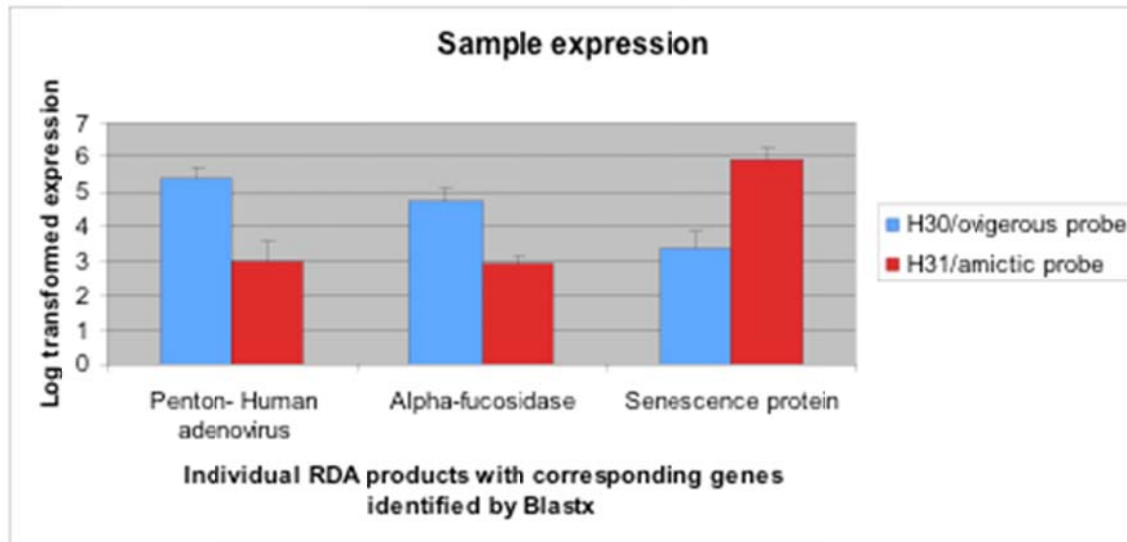
**Experimental methodology on estimation of density at sex initiation in cyclically parthenogenetic rotifers.** Several methodologies have been used in the literature to estimate the population density at sex initiation in rotifers. In this investigation we analyzed the potential effects of the methodology in combination with intrinsic features of the population on the density being reported. We have developed a theoretical model describing the dynamics of both population density and the concentration of the chemical signal triggering sex. The experimental procedures are also simulated. Model analysis showed that the time at which sex is recorded in an experiment, the initial density of the experimental population (usually, culture volume) and the population growth rate can affect the estimation of density thresholds. Results from an experiment in which density at sex initiation was tested for 29 clones of *Brachionus plicatilis* at two culture volumes, revealed that density threshold at which sex initiation is observed is negatively dependent on the experimental volume. We conclude that in order to estimate density thresholds so that they are comparable across studies, experimental methodology should be standardized.

### **Differential Gene Expression in Amictic and Mictic Females**

A supplemental award of NSF Biocomplexity grant (#0412674) to M. Morgan at Berry College.

In October 2007, the Morgan lab began isolating the RNA from the biomass of two rotifer populations represented as: 1) Amictic females filtered from an early log phase culture, and 2) Ovigerous females filtered from a late log phase culture –containing amictic and mictic females. In April 2008, we isolated approximately 576 sequences and recently confirmed that a total of 623 sequences have been isolated and sequenced for this project. A contig assembly program has identified a total of 282 unique sequences which represents 254 singletons and 26 contigs. Glycerol stocks have been made for all cloned sequences. Blast X searches revealed that approximately 70% of the RDA fragments showed homology to genes in GenBank with E-values ranging from 4.00E-55 to 9.3. The RDA products that aligned with known genes were grouped according to KEGG classifications into subgroups including Cell Communication, Apoptosis, Oxidative Phosphorylation, and Transcription Factors.

Reverse Northern blots were used as a preliminary screening to putatively identify those RDA products which appear to be differentially expressed. Of the sequences showing significant homology to known genes, 155 RDA products have been screened using Reverse Northern blots. All expression data was measured with densitometry,  $\text{Log}_2$  transformed, and then statistically analyzed using Student t-tests to determine if there were any significant differences in the expression levels of individual genes within the two populations. Sixteen RDA products exhibited significant differences ( $p < 0.05$ ) in levels of expression between the exclusively amictic and the mixed amictic & mictic populations (Figure 6).



**Figure 6.** A representative sample of virtual Northern expression for three RDA products which had significant differences ( $p < 0.05$ ) in expression levels between the two populations.

An additional 20 RDA products had dot blot signals that showed the potential for differential expression because their p-values were very near the 0.05 threshold. Northern dot blots were performed on these 36 RDA products to confirm differential expression. The work performed to date has provided six Berry College undergraduates with significant training in molecular biology.

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